Electrophysiological and Pharmacological Analysis of Associative Olfactory Learning in the Cockroach

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論文内容の要旨

To fulfill basic demands of life, animals have acquired ability to learn and memorize information essential for them. Elucidation of the neural basis for learning and memory is one of the central themes in neuroscience. Insects are pertinent models to address this question, for their high learning ability and simple, accessible brains with fewer neurons identifiable across individuals. Most previous studies of insect learning have focused on elemental associative learning between the conditioned stimulus (CS) and unconditioned stimulus (US), which represents a process to link directly a specific stimulus to another. Since higher animals encounter many problems to be solved with the aid of a non-elemental form of associative learning, it is mandatory to develop a model for this form of association in simpler nervous systems. The aim of this thesis is to establish such an experimental model and reveal mechanisms for non-elemental learning in a cockroach, *Periplaneta americana*. They have high learning ability to associate an olfactory CS with a gustatory US in both operant and classical conditioning, and olfactory classical conditioning can be monitored in immobilized animals by observing activity changes recorded from salivary neurons. In addition, olfactory processing pathways have been characterized anatomically and physiologically at the single-cell level in the cockroach brain, taking advantage of their relatively large size and accessible organization.

First, I studied the capability of context-dependent learning, a variant of non-elemental learning, in free moving cockroaches. The context-dependence of the paradigm requires a particular type of conditional learning, in which a stimulus (e.g., odor) has different associates with reward reinforcement depending on the conditions (contexts) where it is presented. In the present experiment, a group of cockroaches received training to associate peppermint (CS) with sucrose (appetitive US, US+) and vanilla (CS) with sodium chloride (aversive US, US-) under illumination and to associate peppermint with US- and vanilla with US+ in the dark (Fig. 1). Another group received training with the opposite stimulus arrangement. Before training, both groups exhibited preference for vanilla over peppermint. After training, the former group preferred peppermint over vanilla under illumination but preferred vanilla over peppermint in the dark, and the latter group exhibited the opposite odor preference. I concluded that cockroaches are capable of disambiguating the meaning of CSs according to the visual context.

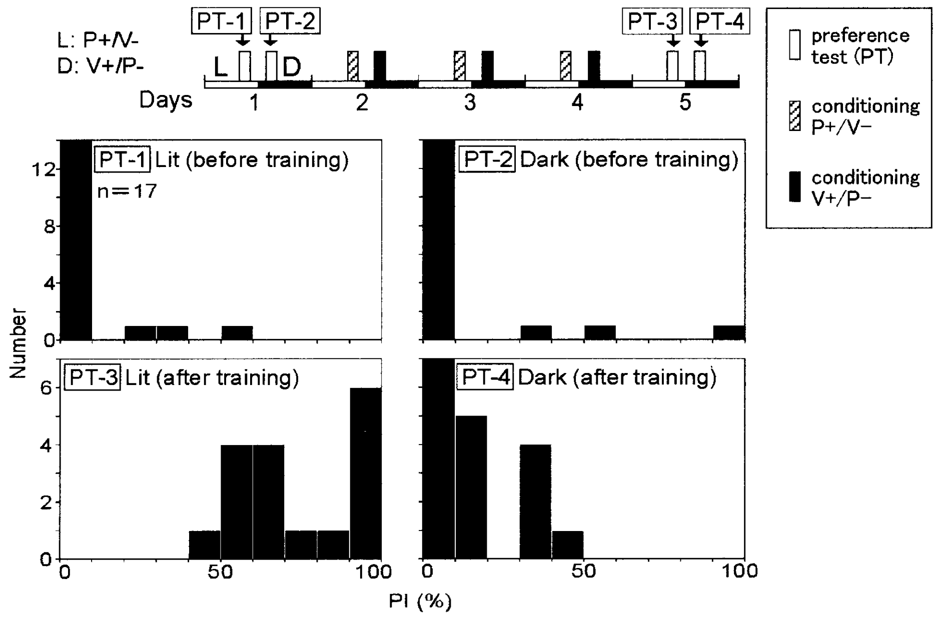


Fig. 1. Effects of context-dependent olfactory conditioning trials. The diagrams at the top show the time schedules for the odor preference test (PT, white bars) and training (shaded and black bars). The white and black parts of the horizontal time bar indicate photophase (12 h) and scotophase (12 h), respectively. Cockroaches were subjected to P−/V+ conditioning trials (shaded bars) under illumination and P+/V− conditioning trials (black bars) in the dark. The upper two histograms show the distributions of preference index for peppermint for each individual in tests conducted before training in a lit condition (PT−1, left) and in a dark condition (PT−2, right), and the lower two histograms show those tested under illumination (PT−3) and in the dark (PT−4) at 1 day after training. The number (n) indicates sample size.

Second, I report that a variant of context-dependent olfactory learning can be demonstrated to occur by extracellular activity recordings from salivary neurons. Restrained cockroaches were trained to associate one of a pair of odors (peppermint or vanilla; CS) with sucrose reward (US+) and the other odor without reward under the flickering light condition (1.0Hz), while they were asked to associate the reversed contingency under the steady light condition. After training, the response to the rewarded odor was significantly greater than that to the unrewarded odor under both light conditions (Fig. 2). I concluded that cockroaches have the capability to learn the reversed CS-US contingency according to the visual context even if they are immobilized, as manifested by

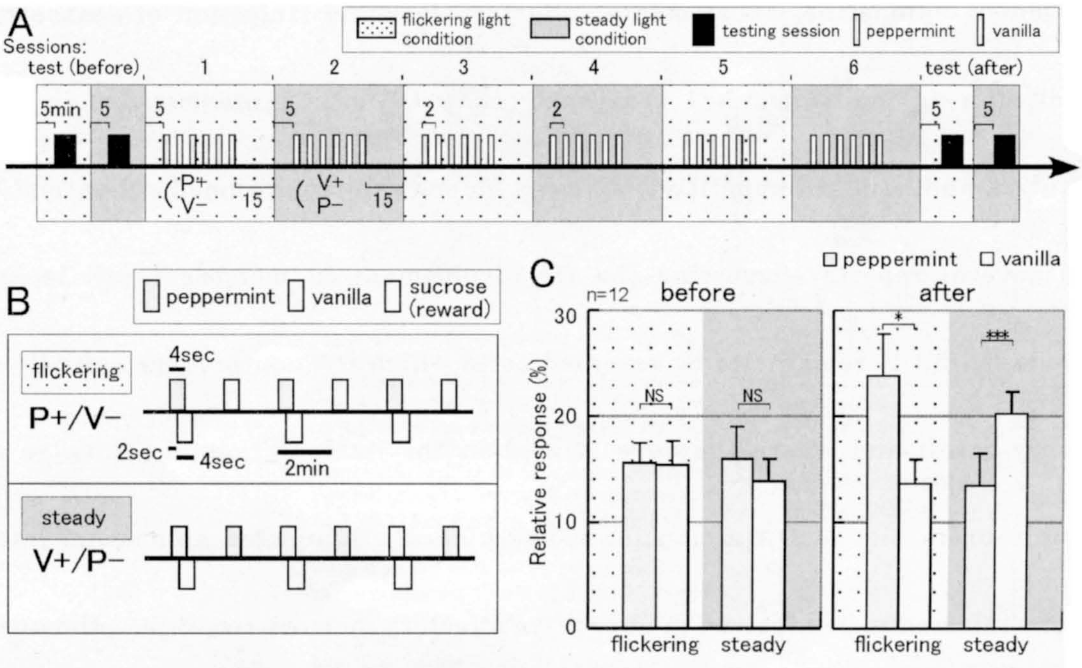
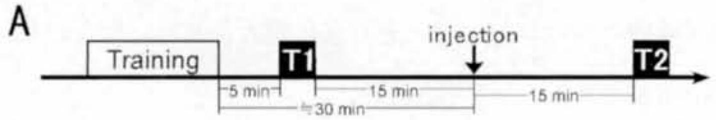


Fig.2. Effects of context-dependent olfactory conditioning trials in group A. A shows the time schedules for the odor preference test (PT, black boxes) and training (blue bars: peppermint trials, yellow bars: vanilla trials). The dotted and gray background boxes in a row indicate the flickering and steady light conditions, respectively. Cockroaches in group A were subjected to P+/V− conditioning trials under flickering light and P−/V+ conditioning trials under steady light. B shows the time course for one session of training in each light condition. C shows the responses of salivary neurons to peppermint odor (blue, Rp) and to vanilla odor (yellow Rv) under flickering light and steady light conditions before and after training. Data are shown by means + S.E. The results of statistical comparisons are shown by asterisks (paired t-test, **P < 0.01, *P < 0.05; NS P > 0.05). The number (n) indicates sample size.

the activity changes in salivary neurons. The findings that cockroaches manage the context-dependent task relying on differential illumination patterns demonstrate high capabilities of cockroaches to perform non-elemental learning.

Third, using the extracellular recording paradigm, I studied the roles of octopamine (OA), a biogenic amine implicated in mediating reward signals in the formation of elemental appetitive olfactory memory in insects. Given that OA-ergic transmission conveys the reinforcement signal in cockroach elemental appetitive learning, then it may as well be involved in context-dependent olfactory learning to achieve an analogous function. To test this, I examined the effect of systemic (global) and local injections of an OA receptor antagonist, epinastine, on elemental olfactory learning. Injection of epinastine into the hemolymph (global injection) confirmed that OA-ergic transmission is required for formation and recall of appetitive olfactory memory for conditioned salivation. There have been several reports suggesting the OA-involvement in memory recall in crickets and honeybees, but it remains to be revealed as to which OA neurons are actually involved in memory recall and where they are located in the nervous system. Thus, to explore the possible brain site of OA action for memory recall, I injected epinastine into the brain regions inferred to be involved in appetitive olfactory memory recall, i.e., the antennal lobes (ALs), calyces and vertical lobes of the secondary olfactory neuropil mushroom body (MB). Indeed, these regions constitute olfactory pathways and known to show distinct OA-like immunoreactivity in cockroaches. In particular, the crucial role of MBs for the formation of olfactory memory has been proposed in cockroaches, yet roles of MB OA-ergic transmission for memory recall have remained to be elucidated. On the other hand, ALs are suggested to be involved in olfactory memory formation and recall in honeybees but not in cockroaches. I injected locally epinastine into the MB vertical lobes, MB calyces or ALs after conditioning, and demonstrated that OA-ergic neurons in the MB vertical lobes (Fig. 3), but not in MB calyces or ALs, participate in memory recall for conditioned salivation. The result is the first to unveil the role of OA-ergic transmission in MB lobes in appetitive olfactory memory recall. This study provides a clue to the neurochemical mechanism for context-dependent learning and memory recall in a simple nervous system that is amenable to sophisticated manipulations for further neurobiological analysis.

A



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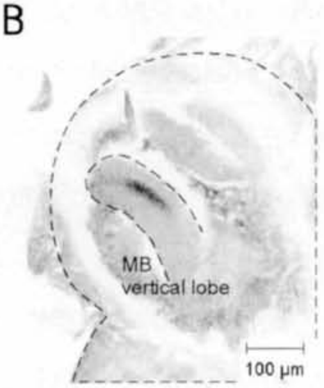

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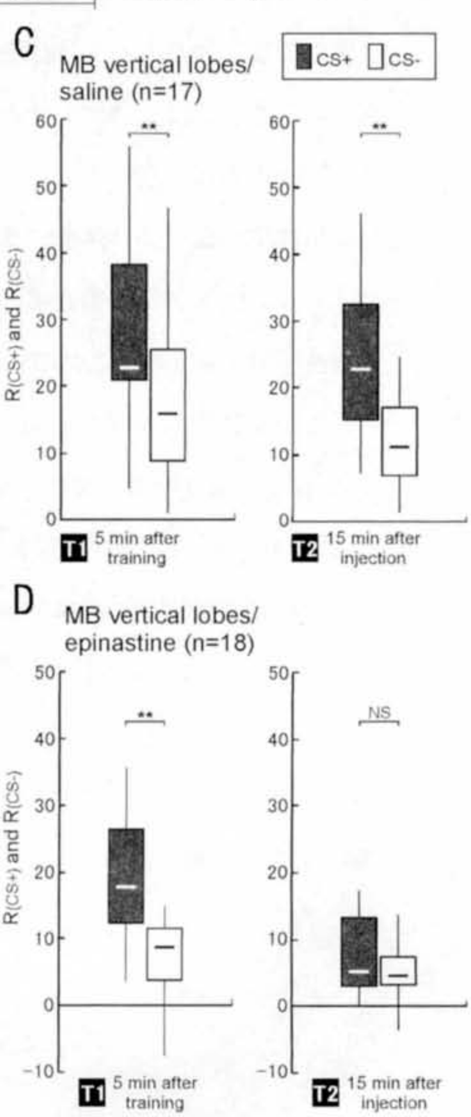

Fig.3. Effects of epinastine local injection into MB vertical lobes.
(A) Experimental schedule. (B) Confocal image of Lucifer Yellow (0.1%) distribution contained in the solutions injected into MB vertical lobe (frontal view). (C) Increase of responses to CS+ or CS- odors in saline injected group. C. Increase of responses to CS+ or CS- odors in 10 μM epinastine injected group. Two groups of cockroaches were injected with 0.2 nl of saline (C) or saline containing 10 μM epinastine (D) into the MB vertical lobes. Both groups were subjected to five "CS+ -sucrose" trials and five "CS- -alone" trials in pseudo-random orders in the training session. Half of the cockroaches in both groups were trained with peppermint odor CS+ and vanilla odor CS-, and the other half vice versa. Responses to CS+ (gray boxes) and those to CS- (white boxes) 5 min after training (test 1) and 1 hour after injection (test 2) are shown as box and whisker diagrams. Outliers are not shown in diagrams, but are included for data analysis. Responses to CS+ were compared with those to CS- in each test. The results of statistical comparisons are shown as asterisks (WCX-test, $^{**}P < 0.01$, $^{*}P < 0.05$; NS $P > 0.05$)

論文審査結果の要旨

学習と記憶のメカニズムの解明は神経科学の中心課題の一つである。比較的単純な神経構造を持ちながら高次な学習をこなす昆虫を用いると、効率的で精度のよい解析が期待できる。そうした観点からワモンゴキブリを材料に選び、匂いと餌の古典的条件付け課題を遂行させて、その際に働く強化神経経路の解明を目指した。条件付けの過程で生じる神経活動の記録を可能にするため、“不動”の状態で学習させるシステムの構築も行った。

これまでの研究に基づき、ペパーミントとバニラの匂いを条件刺激に用い、そのいずれかを報酬のショ糖、すなわち無条件刺激と連合させるパラダイムで学習させた。条件付けの評価は、行動反応によってではなく唾腺ニューロンのインパルス発火頻度の変化に基づいて行った。

ゴキブリはトレーニングの後、ショ糖を与えずに行うテスト試行において、条件刺激の提示に応じて唾腺ニューロンの発火を有意に増大させた。すなわち、不動化した状態で神経活動の条件付けが可能であることが示された。

昆虫では、オクトパミン作動性ニューロンが報酬系として働くと提唱されている。そこで、上記のパラダイムにおいて、オクトパミン受容体阻害剤エピナスティンを血リンパに投与したところ、記憶の形成と読み出しが共に障害された。さらに脳への局所注入実験から、エピナスティンの作用部位が脳キノコ体垂直葉であると推定された。この領域に終末を持つオクトパミン作動性ニューロンがキノコ体垂直葉の内在性介在ニューロン（ケニヨン細胞）に働きかけ、記憶を生む可能性が示唆される。この領域に注目した神経接続の形態的・機能的研究が今後の課題である。

上記の不動化ゴキブリを用いた基本的な条件付けに加えて、課題遂行時の照明条件(context)に依存して条件刺激の意味（報酬の手掛かりとなるか否か）が変化するパラダイムを開発し、ゴキブリが context 依存的学習を行うことを実証した。したがって、とりわけ高度な学習とされる context 依存的学習の機構を解明する上でも、ゴキブリは優れたモデルになると期待される。

このように、佐藤千尋は独自の実験システムを構築し、それを用いて学習の神経機構解明に新たな道筋をつけた。このことは、佐藤千尋が自立して研究活動を行うに必要な高度の研究能力と学識を有することを示している。したがって，佐藤千尋提出の論文は，博士（生命科学）の博士論文として合格と認める。